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Armadillo Coactivates Transcription Driven by the Product of the Drosophila Segment Polarity Gene *dTCF*

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Summary

The vertebrate transcription factors TCF (T cell factor) and LEF (lymphocyte enhancer binding factor) interact with β-catenin and are hypothesized to mediate Wingless/Wnt signaling. We have cloned a maternally expressed Drosophila TCF family member, *dTCF.* dTCF binds a canonical TCF DNA motif and interacts with the β-catenin homolog Armadillo. Previous studies have identified two regions in Armadillo required for Wingless signaling. One of these interacts with dTCF, while the other constitutes a transactivation domain. Mutations in *dTCF* and expression of a dominant-negative *dTCF* transgene cause a segment polarity phenotype and affect expression of the Wingless target genes *engrailed* and *Ultrabithorax*. Epistasis analysis positions *dTCF* downstream of *armadillo*. The Armadillo–dTCF complex mediates Wingless signaling as a bipartite transcription factor.

Introduction

Wingless/Wnt signaling directs the establishment of segment polarity in Drosophila melanogaster and controls several key developmental decisions in vertebrates. The fly Wingless signaling pathway is strikingly similar to the vertebrate Wnt signaling cascade, which controls dorsal–ventral patterning in Xenopus laevis (Klymkowsky and Parr, 1995). The segment polarity gene *armadillo* encodes the most downstream known component in the Wingless signaling pathway (reviewed in Peifer, 1995). Armadillo and its vertebrate homolog β-catenin accumulate inside cells in response to Wingless/Wnt signals. They also occur in adherens junctions complexed with cadherin homologs. Armadillo and β-catenin consist of 13 Armadillo (arm) repeats flanked by unique N- and C-termini. Genetic and biochemical studies have revealed the regions essential for interaction with Armadillo's adherens junction partners (Orsulic and Peifer, 1996a; Cox et al., 1996; Pai et al., 1996). The C-terminus of Armadillo is dispensable for adhesion but essential for Wingless signaling (Peifer et al., 1994b). A second essential region for Wingless signaling maps to the central repeats 3–8 of Armadillo (Orsulic and Peifer, 1996a).

Members of the TCF/LEF (T cell factor/lymphocyte enhancer binding factor) family of transcription factors, specifically Xenopus XTcf-3 (Molenaar et al., 1996) and murine LEF-1 (Behrens et al., 1996; Huber et al., 1996), physically interact with β-catenin. XTcf-3 in isolation binds DNA but requires β-catenin to activate transcription (Molenaar et al., 1996). Injection of β-catenin into early Xenopus embryos induces the formation of a secondary axis (Funayama et al., 1995), mimicking the effect of Wnt activation. Ectopic expression of a dominant-negative form of *XTcf-3*, lacking the region required for β-catenin binding, blocks β-catenin's ability to cause axis duplication and blocks formation of the endogenous axis (Molenaar et al. 1996). Similar observations were made with murine *LEF-1* (Behrens et al., 1996; Huber et al., 1996). These data imply that vertebrate TCF/LEF factors transduce Wnt signals. We have now analyzed the role of a Drosophila TCF/LEF family member, *dTCF*, in Wingless signaling.

Results

Cloning and Chromosomal Mapping of *dTCF*

Using a polymerase chain reaction (PCR)–based strategy, we cloned a single *TCF* homolog, termed *dTCF*, highly related to the vertebrate *TCF* genes and to Caenorhabditis elegans *pop-1* (Lin et al., 1995; Figure 1). Three regions of conservation were noted. First was the N-terminus, which in XTcf-3 and LEF-1 constitutes the β-catenin interaction domain. Second was the high mobility group (HMG) box DNA-binding domain. Infrequently, an alternative exon encoding the second part of the HMG box was encountered. The alternative protein was termed dTCF-B. Third, a small region directly C-terminal to the HMG box, was conserved among dTCF, TCF-1 (van de Wetering et al., 1996), and pop-1.

By in situ hybridization to polytene chromosomes, *dTCF* maps to the centromere proximal region of the fourth chromosome in section 101F, close to the segment polarity gene *cubitus interruptus* (*ci*) (Orenic et al., 1990; Locke and Tartoff, 1994). Restriction mapping suggested a head-to-head orientation of *ci* and *dTCF*, confirmed by sequencing of the *ci* cosmid 4–1 (Orenic et al., 1990) (Figure 2).

# These authors contributed equally to this study.

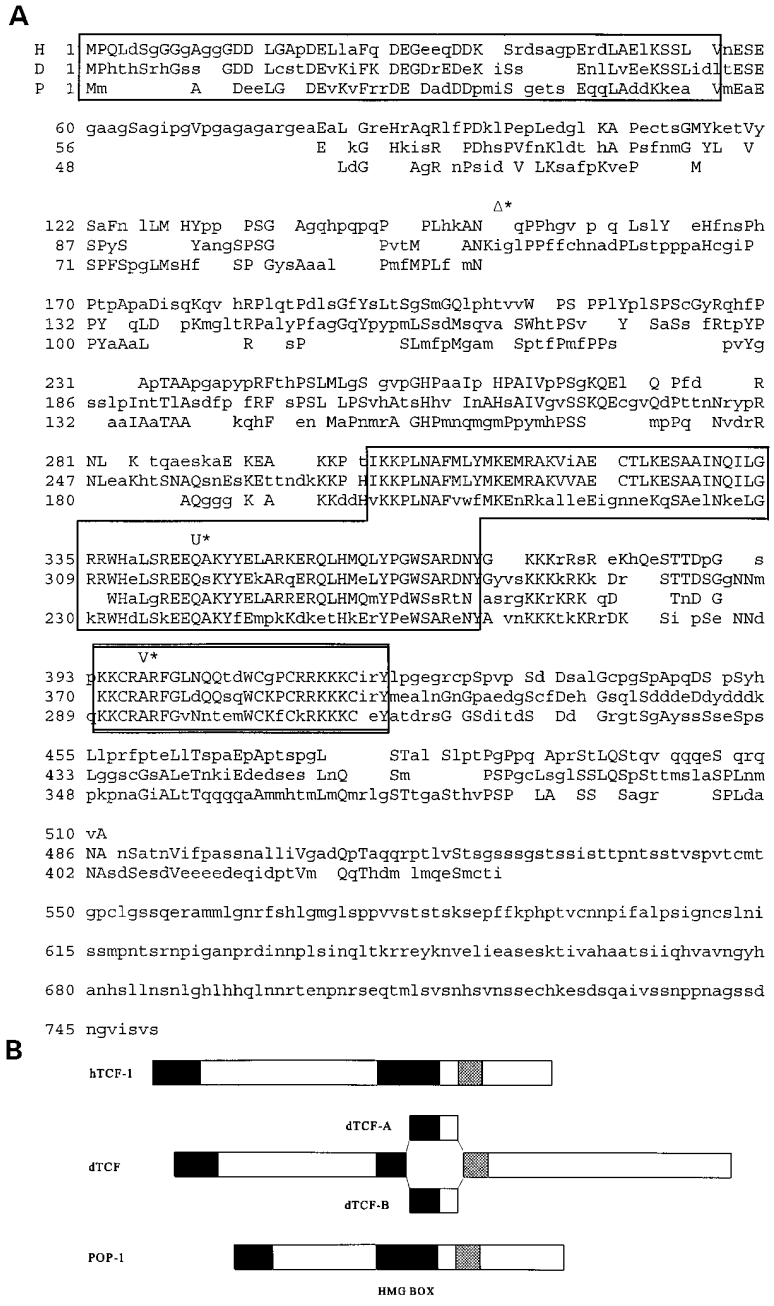

Figure 1. *dTCF* Is Similar to Vertebrate and *C. elegans TCF* Genes

(A) Sequence comparison of *dTCF* (D) to C. elegans *pop-1* (P) and human *TCF-1E* (H). Boxed sequences represent the minimal N-terminal β-catenin/Armadillo interaction domain and the DNA-binding HMG box. The two isoforms dTCF-A and dTCF-B differ in the second half of the HMG box of dTCF. The double box indicates a third homology domain, which contains the *dTCF*[1] mutation. Δ*, the *dTCF*[2] single-base deletion (ATT to AT); U*, the CAA-to-TAA mutation in *dTCF*[3]. GenBank accession number: Y09125.

(B) Diagrams comparing the structures of hTCF-1, the alternate splice forms of dTCF, and pop-1.

Maternal Expression of *dTCF*

By reverse transcription PCR, we demonstrated expression of *dTCF* at all stages of development. *dTCF-A* mRNA was already found in embryos of 0–2 hr, indicative of maternal expression. *dTCF-B* was not expressed during embryogenesis (Figure 3A). Whole- mount in situ hybridization revealed high levels of maternal mRNA in early cleavage stage embryos (Figure 3B). At the end of germband extension, most tissues expressed high levels of *dTCF* (Figure 3C). By the end of germband extension, embryos homozygous for a deletion removing *dTCF* and adjacent genes had lost their maternal *dTCF* mRNA (Figure 3D).

dTCF Physically Interacts with Armadillo

The N-termini of XTcf-3 and LEF-1 interact with the Armadillo repeat region of β-catenin (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996). In the yeast two-hybrid system, repeats 3–8 of Armadillo interacted with amino acids 1–90 of dTCF (Figure 4). Deletion of the N-terminal 31 amino acids of dTCF abrogated the interaction. Essentially identical data were obtained for the interaction of Armadillo with XTcf-3.

Certain mutations in the repeat region of Armadillo eliminate its ability to transduce Wingless signals in vivo (Orsulic and Peifer, 1996a). The effects of such mutations on Wingless signal transduction and on dTCF bind-

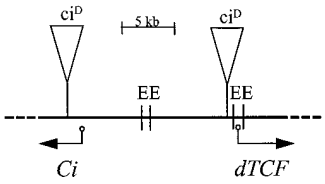

Figure 2. Organization of the Promoter Region of *ci* and *dTCF*

The indicated *ci^D* insertions were originally described by Locke and Tartoff (1994). E, EcoRI.

ing correlated well. The *arm^S11* mutation, which deletes repeats 3–6 and eliminates Wingless signaling, completely blocked dTCF binding. The *arm^S12* and *arm^S6* mutations, which alter repeats 1 and 10–11, respectively, have only subtle effects on Wingless signaling in vivo. These two mutations did not affect dTCF binding.

Armadillo Is a Coactivator of dTCF-Driven Transcription

To determine the optimal DNA-binding site of dTCF, we performed PCR-based binding site selection using a fragment of dTCF spanning the HMG box. Sequencing of 36 selected binding motifs revealed the consensus $C_{36}C_{36}T_{36}T_{36}T_{36}G_{36}A_{36}T_{36}C_{33}T_{34}T_{34}$ (where numbers in subscript represent the frequency of the indicated base), matching well with the canonical TCF binding motif CCTTTGA/TA/T (van de Wetering et al., 1991, 1993).

TCF factors do not activate transcription from promoters containing multimerized TCF binding sites (van de Wetering et al., 1993). Cotransfection of XTcf-3 with β-catenin resulted in transcriptional activation (Molenaar et al., 1996). Cotransfection of XTcf-3 with Armadillo had similar effects (Figure 5A). Importantly, Armadillo and β-catenin were capable of coactivated transcription driven by dTCF. Similar levels of cotransactivation were observed for two mammalian TCF factors: human TCF-1 and LEF-1. The C-terminus of β-catenin was necessary for the effect, whereas its N-terminus was dispensible (Figure 5B). We grafted the C-termini of β-catenin and of Armadillo onto a GAL4 DNA-binding domain and thus demonstrated that these regions constitute genuine transactivation domains (Figure 5C).

Genetic analyses of Armadillo have demonstrated that C-terminal truncation at amino acid 750 (mutant *arm^S8*) leaves the signaling properties of Armadillo intact, while truncation at amino acid 681 (mutant *arm^XM19*) abrogates signaling (Peifer et al., 1994b; Orsulic and Peifer, 1996a). The observed coactivator activities of the deletion mutants correlated well with these genetically determined signaling requirements (Figure 5B). These data, summarized in Figure 5D, underscore the model in which Wingless/Wnt signaling through Armadillo/β-catenin involves the transcriptional coactivation of TCF target genes (Figure 5E).

dTCF Is a Segment Polarity Gene

The *ci* region contains two lethal complementation groups, *l(4)13* and *l(4)17*, both of which fail to complement the mutation *ci^D* (Locke and Tartoff, 1994; Table 1). One lesion in the *ci^D* chromosome maps within the *ci* gene, while the other maps to the promoter of *dTCF* (Figure 2). *l(4)17* is clearly equivalent to *ci* (Locke and Tartoff, 1994; Slusarski et al., 1995). Mutants in *l(4)13* thus represented candidates for *dTCF* mutations. We sequenced the *dTCF* gene in the only extant allele, *l(4)13a* (Hochman, 1971), and found a single missense mutation (C to T; A374V) (Figure 1).

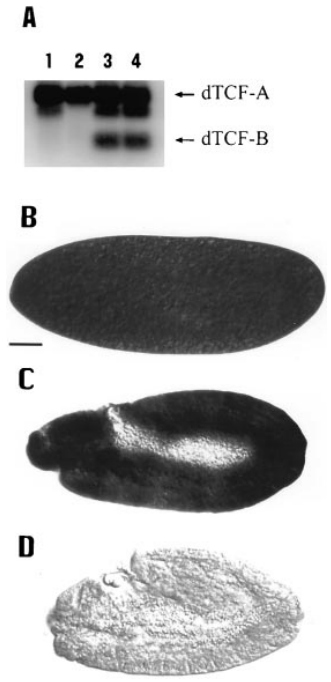

Figure 3. Maternal Expression of *dTCF-A*

(A) Reverse transcription PCR analysis for *dTCF-A* and *dTCF-B* expression in embryos (0–2 hr, lane 1; 2–16 hr, lane 2; adult female, lane 3; and adult males, lane 4. Primers spanned dTCF from amino acids 212–402. The PCR product was digested with SacI (unique to *dTCF-B*) and Southern blotted. Probing with a fragment encoding amino acids 367–402 reveals the two isoforms in a quantitative fashion. Only *dTCF-A* was expressed at the embryonic stages.

(B–D) In situ hybridization of wild-type (B and C) and homozygous *Df(4)M62f* (negative control) (D) embryos. (B) Cleavage stage; (C) germband-extended stage; (D) similar stage as the embryo in (C). No *dTCF* mRNA is visible. Bar, 50 μM.

Transgenic lines carrying a *dTCF-A* cDNA under the control of a heat shock promoter were generated. Flies carrying the transgene did not display detectable phenotypic abnormalities. Three independent lines were tested for their ability to rescue homozygous *l(4)13a* flies. *l(4)13a/ey^D* flies carrying one or two copies of a given rescuing transposon were mated to each other and their progeny were examined (Table 1). The low level expression at 25°C of all three tested lines was sufficient to allow *l(4)13a* homozygotes to survive to the adult stage. We concluded that *l(4)13a* results from a defect in the *dTCF* gene. The mutation was renamed *dTCF^1*.

In an unrelated screen (A. B., unpublished data), we obtained two mutations with a segment polarity phenotype. Both map to the fourth chromosome; complement

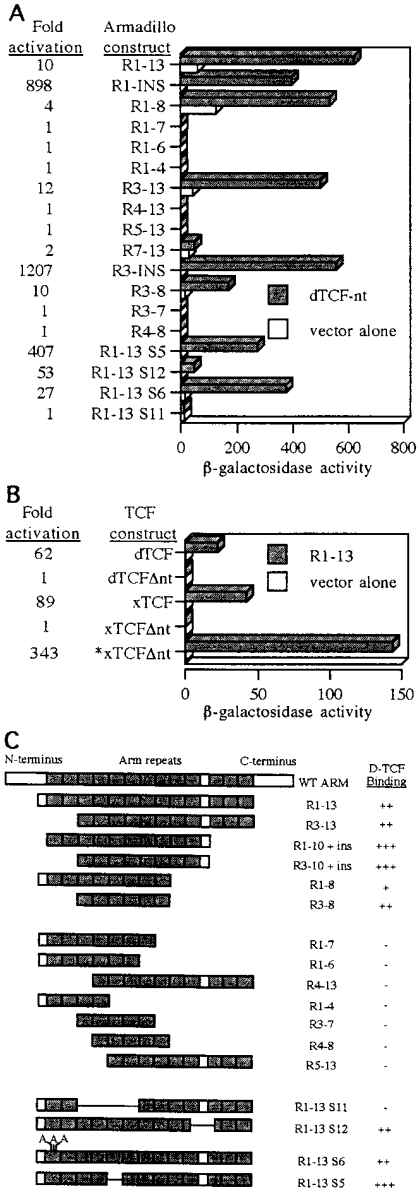

Figure 4. The N-Terminus of dTCF Interacts with Repeats 3–8 of Armadillo

(A) Various portions of Armadillo fused to the LexA DNA- binding domain were tested in a two-hybrid analysis with either the N-terminal 90 amino acids of dTCF (dTCF-nt) fused to the GAL4 transactivation domain (in pCK4) or the pCK4 vector alone. Fold activation is the ratio of the β-galactosidase activity obtained using dTCF-nt and that obtained using pCK4 only.

(B) Full-length dTCF (amino acids 1–759), dTCFΔnt (amino acids 32–759), full-length XTcf-3 (amino acids 1–559), and XTcf-3Δnt (amino acids 32–559) fused to the LexA DNA-binding domain in pCK2 were tested with arm repeats 1–13 (R1–13) of Armadillo fused to the GAL4 transactivation domain in pCK4 or with pCK4 alone. *XTcf-3Δnt is the reciprocal interaction tested (amino acids 32–559 of XTcf-3 fused to the GAL4 transactivation domain tested against R1–13 fused to the LexA DNA-binding domain or pCK2 alone.

(C) Summary of (A). −, ratio of <2; +, ratio of 2–9; ++, ratio of 10–100; +++, ratio of >100.

a null mutation in *ci*; and fail to complement *dTCF*^{1}, *ci*^{D}, and one another. These new alleles of *dTCF* were subsequently sequenced. In *dTCF*^{2}, loss of a base pair led to a frameshift (ATT to AT at I106) (Figure 1). In *dTCF*^{3}, a CAA-to-TAA mutation introduced a stop codon at Q319 in the HMG box of dTCF-A. *dTCF*^{2} flies should not make any functional dTCF protein. *dTCF*^{3} flies can still potentially produce dTCF-B protein at later stages of development. Both *dTCF*^{2} and *dTCF*^{3} flies had a segment polarity phenotype resembling that of a moderate *armadillo* mutation like *arm*^{TD}, which lacks the Armadillo C-terminus (Figures 6B–6F; Peifer and Wieschaus, 1990). In the abdominal segments, most surviving cells chose denticle fates; there sometimes was a small amount of naked cuticle along the ventral midline. The head segments were relatively unaffected. These *dTCF* alleles behaved as genetic nulls: their phenotype did not become more severe when heterozygous with a deletion removing *dTCF*, *Df(4)M62f* (Figures 6C and 6E). This was consistent with the observed molecular lesions. The *dTCF* zygotic null phenotype was not as severe as that of a null mutation in *wingless* (Figure 6O), presumably because of the substantial maternal contribution of *dTCF*. Our null alleles had a phenotype similar to that described for the other original *dTCF* allele, now lost (Wieschaus et al., 1984; E. Wieschaus, personal communication). *dTCF*^{1} homozygotes died primarily as first instar larvae; a few failed to hatch. They had a weak segment polarity phenotype (Figure 6G): regions of naked cuticle, both anterior and posterior to the normal denticle belt, were replaced by cuticle with denticles. The *dTCF*^{1} phenotype resembled that of weak alleles of *armadillo* such as *arm*^{S6}, which is mutated in the central arm repeats (Orsulic and Peifer, 1996a). *dTCF*^{1} behaved genetically as a hypomorph, as its phenotype became more severe (Figure 6H) over *Df(4)M62f*. The *dTCF*^{1} phenotype was also more severe over *ci*^{D}, consistent with a disruption of *dTCF* on the *ci*^{D} chromosome (Figure 6I).

In *wingless* mutants, *engrailed* expression comes on normally but fails to be maintained (DiNardo et al., 1988). In a null *dTCF* mutant, *engrailed* expression was initiated normally, but the stripes of *engrailed* expression began to decay by late stage 9, particularly in midlateral regions (Figures 7A and 7B) and along the ventral midline. This effect resembled that of a zygotic *armadillo* mutation (Peifer et al., 1991) or of removal of functional Wingless at the end of stage 9 (Bejsovec and Martinez-Arias, 1991).

Mutations in the Wingless pathway also affect the expression of *Ultrabithorax* (*Ubx*) in the visceral mesoderm (Thuringer and Bienz, 1993; Yu et al., 1996), disrupting the secondary midgut constriction (Panganiban et al., 1990; Hursh et al., 1993). Indeed, *Ubx* expression was not maintained in *dTCF*^{2}, while the secondary midgut constriction was absent (Figures 7D and 7E). The primary constriction was not affected and did not move posteriorly. This contrasted with other mutations that disrupt the secondary midgut constriction (Panganiban et al., 1990; Hursh et al., 1993), indicating that the *Ubx* regulatory network might be only partially disrupted by *dTCF*^{2}.

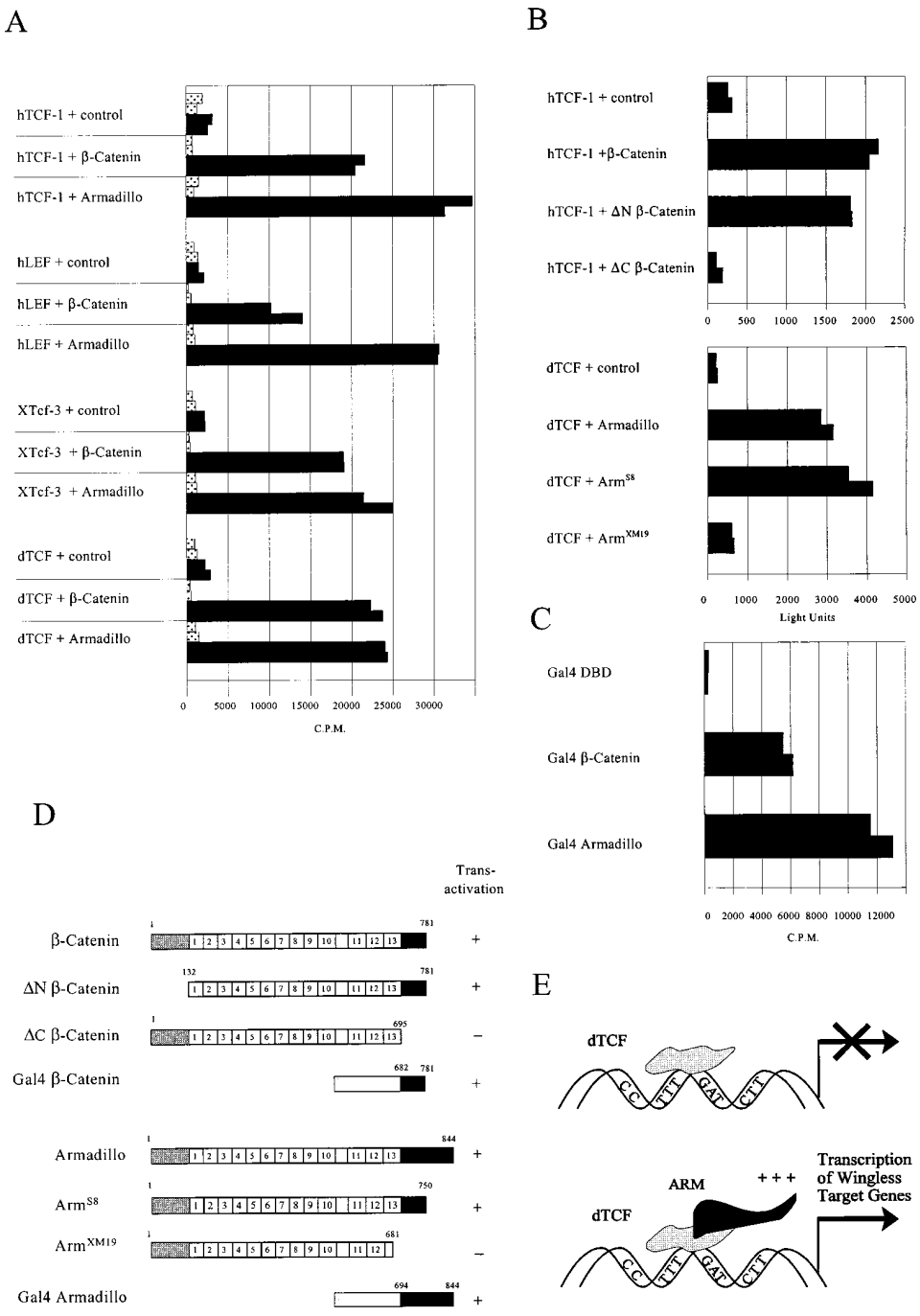

Figure 5. Cotransactivation of TCF-Driven Transcription by the C-Termini of β-Catenin and Armadillo

(A) Cells were transfected either with a CAT vector containing an minimal promoter and an upstream concatamer of the TCF/LEF cognate motif ($pTK(56)_7$) or the negative control vector ($pTK(56Sac)_7$). Cotransfections were performed with the indicated expression plasmids. Relative CAT activity is presented as counts per minute (CPM). Both values of duplicate transfections are given.

(B) Cells were transfected with pTOPFLASH (containing a concatamer of the TCF/LEF cognate motif). Both β-catenin (top) and Armadillo (bottom) require their C-terminus for cotransactivation in the context of human TCF and dTCF, respectively.

(C) Cells were transfected with the $p(GAL4)_2$ TK-CAT reporter construct and with vectors expressing the indicated GAL4 fusion proteins. The C-termini of β-catenin and of Armadillo constitute transactivation domains in the context of the GAL4 DNA-binding domain.

(D) Summary of cotransactivation results.

(E) Schematic representation of the proposed molecular model.

Table 1. Rescue of *l(4)13a* by Different *hsdTCF*$^{+}$ Lines

Transgenic Line	Number of Progeny			% of Expected
	l(4)13a/ey^{D}	*l(4)13a/l(4)13a*	Expected	
—	77	0	38.5	0
$hsdTCF^{+}_{15\text{-}X}$	84	27	42	64
$hsdTCF^{+}_{15\text{-}2}$	92	13	46	28
$hsdTCF^{+}_{15\text{-}32}$	66	23	33	70

Dominant-Negative dTCF Mutations Disrupt the Wingless Signal

A mutant XTcf-3 lacking the N-terminal β-catenin interaction domain has dominant-negative effects on axis formation in Xenopus (Molenaar et al., 1996). We expressed a similar N-terminal deletion mutant of *dTCF* (*dTCF*Δ*N*) in flies under the control of an inducible promotor, using the GAL4-UAS system. Ubiquitous expression of *dTCF*Δ*N* in a wild-type embryo beginning late in stage 9, using either e22-GAL4 or 69B-GAL4, resulted in a segment polarity phenotype (Figures 6L and 6M; five of six lines tested). The severity of this phenotype varied from that of a zygotic null *dTCF* mutation (Figure 6L) to that of a *wingless* null mutation (Figures 6N and 6O). These latter embryos may reflect blockage of both maternal and zygotic dTCF function. In the most severe transgenics, the denticle belts were also narrowed in the dorsal–ventral axis. Ubiquitous expression of *dTCF*Δ*N*, using either e22-GAL4 (data not shown) or 69B-GAL4 (Figure 7C), resulted in a decay of the stripes of *engrailed* expression during late stage 9, in particular in the most posterior abdominal segments. Ubiquitous expression of full-length dTCF did not result in a *wingless*-class segment polarity phenotype. Four of the six lines tested were fully or partially embryonic viable, while two were embryonic lethal. Two of the viable lines had normal cuticle patterns; in the other lines, the denticle belts were slightly narrowed (Figure 6Q).

dTCF Is Required for Armadillo Function

The *armadillo* transgene *arm*^{S10} lacks 54 amino acids in its N-terminus and is constitutively active in Wingless signaling (Pai et al., submitted). Ubiquitous expression of *arm*^{S10} transforms all cells to posterior cell fates, as indicated by the secretion of naked cuticle (Figure 6K). The action of *arm*^{S10} is not affected by upstream mutations in the Wingless cascade, but it should be blocked by alterations in proteins that act with or downstream of *armadillo*. When Arm^{S10} was expressed ubiquitously in *dTCF*^{1} homozygous embryos, its action indeed was largely inhibited. The double mutant embryos (Figure 6J) were distinct from *dTCF*^{1} homozygotes (Figure 6G) and from wild-type embryos expressing Arm^{S10} (Figure 6K and Table 2). They resembled *dTCF*^{1} homozygotes, in that they had alternating denticles and naked cuticle, with portions of the lateral naked cuticle converted to denticles. However, they often had regions of naked cuticle intruding into the normal denticle belt at the ventral midline. We also generated animals expressing *arm*^{S10} in *dTCF*^{2} and *dTCF*^{3} mutant backgrounds. These animals were indistinguishable in phenotype from the

Table 2. dTCF Is Downstream of Armadillo in the Wingless Signaling Pathway

Cross 1 $\frac{e22c\text{-}GAL4.dTCF^{1}}{+};\frac{}{+} \times \frac{arm^{S10}.dTCF^{1}}{+};\frac{}{+}$ n = 273

Genotypes of Progeny	Phenotype	Predicted	Observed
Various genotypes	Wild type	56.3% (9/16)	54.6%
$\frac{arm^{S10}}{e22c}.\frac{+}{dTCF^{1}}$ or +	Naked	18.8% (3/16)	21.2%
$\frac{+}{arm^{S10}}$ or $e22c.\frac{dTCF^{1}}{dTCF^{1}}$	Denticle lawn	18.8% (3/16)	17.9%
$\frac{arm^{S10}.dTCF^{1}}{e22c}.\frac{}{dTCF^{1}}$	Denticle lawn with midline naked	6.3% (1/16)	6.2%

Crosses 2 and 3* $\frac{e22c\text{-}GAL4.dTCF^{x}}{+};\frac{}{+} \times \frac{arm^{S10}.dTCF^{x}}{+};\frac{}{+}$ n = 474 *dTCF*^{2}; n = 507 *dTCF*^{3}

Genotypes of Progeny	Phenotype	Predicted	Observed	
			dTCF^{2}	*dTCF*^{3}
Various genotypes	Wild type	56.3% (9/16)	57.4%	52.5%
$\frac{arm^{S10}}{e22c}.\frac{+}{dTCF^{x}}$ or +	Naked	18.8% (3/16)	19.0%	20.3%
$\frac{+}{arm^{S10}}$ or $e22c.\frac{dTCF^{x}}{dTCF^{x}}$	Denticle lawn	25% (4/16)	23.6%	27.2%
$\frac{arm^{S10}.dTCF^{x}}{e22c}.\frac{}{dTCF^{x}}$				

arm^{S10} was expressed in *dTCF*^{1}, *dTCF*^{2}, and *dTCF*^{3} mutant backgrounds. Hatched and unhatched embryos were scored for their cuticle phenotype. Crosses are diagrammed. The predicted numbers are based on the hypothesis that *dTCF* is downstream of *armadillo*.
* In crosses 2 and 3, *dTCF*^{x} refers to *dTCF*^{2} and *dTCF*^{3}, respectively.

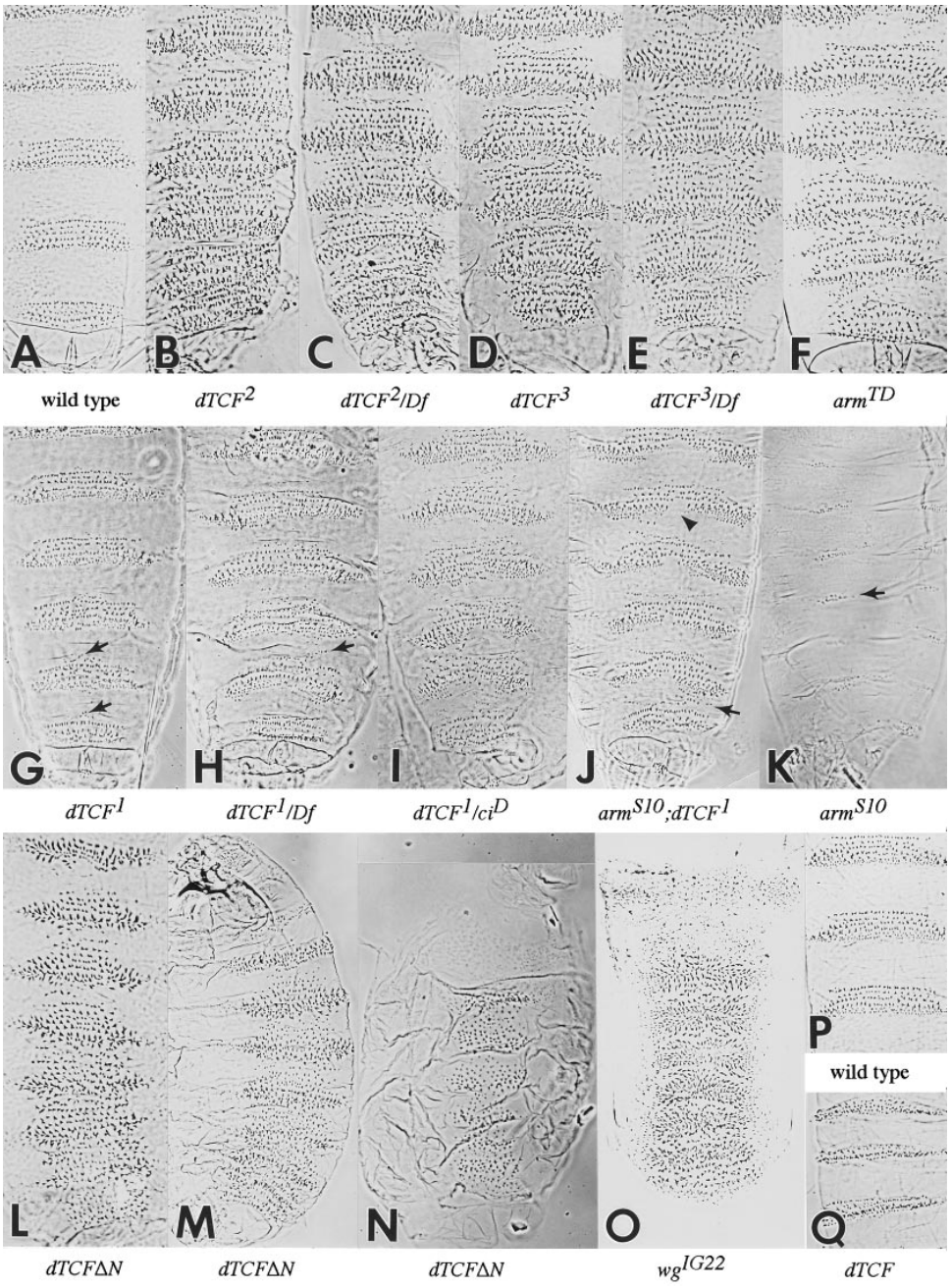

Figure 6. dTCF Is Required for Wingless Signaling In Vivo and Acts Downstream of Armadillo in the Signal Transduction Pathway

Ventral view of cuticle preparations of wild-type and mutant animals.

(A–I) Mutations in *dTCF* have a segment polarity phenotype. (A) In the wild-type embryo, anterior cells of each segment secrete cuticle with denticles, while posterior cells secrete naked cuticle. (B, D, and F) $dTCF^{2}$ and $dTCF^{3}$ homozygous mutant embryos have a phenotype similar to that of arm^{TD}. (C and E) $dTCF^{2}$ and $dTCF^{3}$ over *Df(4)M62f*. (G) $dTCF^{1}$ homozygotes have small patches of ectopic denticles (arrows in G and H). (H and I) $dTCF^{1}$ heterozygous with *Df(4)M62f* and with ci^{D} respectively.

(J and K) $dTCF^{1}$ largely blocks the dominant effects of arm^{S10}. Embryos expressing Arm^{S10} secrete nearly completely naked cuticle (K), with only a few remaining denticles (arrow). arm^{S10};$dTCF^{1}$ (J), with ectopic denticles laterally (arrow) but naked cuticle along the ventral midline (arrowheads).

(L–O) Expression of *dTCFΔN* causes a segment polarity phenotype. (L) *dTCFΔN* line 3, expressed ubiquitously using e22-GAL4. (M) *dTCFΔN* line 4, expressed ubiquitously using e22-GAL4. (N) *dTCFΔN* line 1, expressed ubiquitously using 69B-GAL4. (O) The null allele wg^{IG22} for comparison.

(P and Q) Expression of full-length dTCF has subtle effects on the cuticle pattern. (P) Segments A4–A6 from a wild-type embryo. (Q) Segments A4–A6 from an embryo expressing *dTCF* ubiquitously (line 21, crossed to e22-GAL4).

dTCF single mutants (Table 2; note increased numbers of animals with a lawn of denticles), confirming that *dTCF* mutations block the action of *arm*^{S10}.

Discussion

The current study demonstrates that *dTCF* functions directly downstream of *armadillo* in the establishment of segment polarity and provides a molecular mechanism for gene control by Wingless signaling. The role of *dTCF* that emerges from this study is consistent with the proposed role of *XTcf-3* in axis specification in Xenopus. It thus appears that yet another component is conserved between the Wingless and Wnt signaling pathways.

We can integrate these data into our current picture of Wingless signal transduction (Orsulic and Peifer, 1996b). In the absence of extracellular Wingless, Armadillo accumulates in adherens junctions complexed to cadherins. Levels of uncomplexed, cytoplasmic Armadillo remain low due to its short half-life (Peifer et al., 1994b; van Leeuwen et al., 1994). It is thought that the serine/threonine kinase zeste–white 3 (Peifer et al. 1994b; Siegfried et al., 1994) and possibly a Drosophila homolog of the tumor suppressor adenomatous polyposis coli (Rubinfeld et al., 1996) actively promote Armadillo degradation. The interaction of Wingless with its receptors, members of the Frizzled family (Bhanot et al., 1996), activate the cytoplasmic protein Dishevelled (Yanagawa et al., 1995). This shuts down Armadillo degradation, possibly by way of effects on zeste–white 3 (Cook et al., 1996). Armadillo then accumulates in the cytoplasm and nucleus (Orsulic and Peifer, 1996a) and can bind to dTCF. Thus, an active transcription factor is assembled from two partners: dTCF mediates DNA binding, while the C-terminus of Armadillo activates transcription. Our model predicts that Wingless response elements in regulatory regions of target genes resemble the *dTCF* consensus motif.

The mechanism by which Armadillo enters the nucleus is not fully understood. Armadillo lacks an obvious nuclear localization signal, but Armadillo mutants that cannot bind to dTCF still enter the nucleus (Orsulic and Peifer, 1996a; this study). Both Armadillo and the nuclear import receptor importin are primarily composed of arm repeat motif (Peifer et al., 1994a). Armadillo might directly interact with the translocation machinery of the nuclear pore.

engrailed in the embryonic epidermis (DiNardo et al., 1988; Martinez-Arias et al., 1988) and *Ubx* in the developing midgut (Bienz, 1994) are target genes of the Wingless cascade. A Wingless response element with an essential TCF/LEF binding motif has recently been identified in the *Ubx* promoter (Riese et al., 1997 [this issue of *Cell*]). A fragment of the *engrailed* enhancer acting as a Wingless response element contains a consensus dTCF binding site (Florence et al., 1997). Consistent with our model, mutations in *dTCF* block maintenance of *engrailed* and of *Ubx* visceral mesoderm expression.

The mammalian genome harbors at least four different TCF genes, each with unique expression patterns (Oosterwegel et al., 1993; V. Korinek and H. C., unpublished data). Numerous *Wnt* genes have been identified (Parr and McMahon, 1994). Also, multiple homologs of the Wingless receptor Frizzled-2 (Bhanot et al., 1996) exist in vertebrates (Wang et al., 1996). It is likely that the principles of Wingless signaling as outlined here will apply to a large number of cell fate choices in metazoan development. The similarity of the C. elegans mesoderm-specifier *pop-1* with *dTCF* is tantalizing. Like *dTCF* and *XTcf-3*, *pop-1* is maternally and ubiquitously expressed. *pop-1* specifies the fate of the MS blastomere

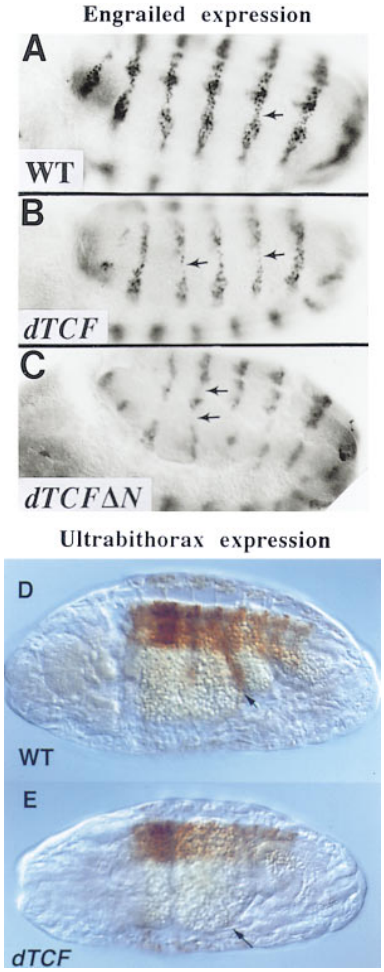

Figure 7. Mutations in *dTCF* Alter Expression of the Wingless Target Genes *engrailed* and *Ubx*

(A) Wild-type engrailed is expressed in the posterior compartment, comprising two to three rows of cells per segment (arrow), beginning before gastrulation and continuing throughout development.

(B) Putative *dTCF*^{3} mutant. Cells in the lateral (e.g., arrows) or midventral (not shown) regions lose engrailed expression during stage 9.

(C) Embryo likely expressing *dTCFΔN* ubiquitously. Engrailed expression is lost in the lateral regions of the more posterior abdominal segments (e.g., arrows), beginning during late stage 9.

(D and E) Ubx staining in wild type (D) and *dTCF*^{2} (E) mutant stage 16 embryos. Arrow in (D) points to the secondary midgut constriction and visceral mesoderm *Ubx* expression. Arrow in (E) indicates the absence of the secondary midgut constriction and Ubx staining. The pattern of ectodermal *Ubx* expression is altered as a consequence of segmental fusions.

at the eight-cell stage (Lin et al., 1995). It is plausible that an Armadillo homolog and possibly other components of the Wingless cascade cooperate with *pop-1* in the determination of MS blastomere fate. In addition to these roles in normal development, this pathway, in which β-catenin accumulation drives formation of bipartite β-catenin/TCF transcription factors, appears to be inappropriately activated in human colon cancer and melanoma (Korinek et al., 1997; Morin et al., 1997; Rubinfeld et al., 1997).

Experimental Procedures

Cloning of *dTCF*

A genomic fragment encoding the HMG box region of *dTCF* was cloned by PCR using degenerate primers, as described elsewhere (Molenaar et al., 1996). Several mixed-stage embryo cDNA libraries were screened with this fragment. Positive clones in pBluescriptSK were sequenced. Genomic sequences were cloned from an EMBL-3 library according to standard procedures.

Fly Stocks, Rescue Constructs, and Phenotypic Analysis

The wild-type stock was Canton S. ci^{D}, ci^{Ce} and *l(4)13a* (Orenic et al., 1987; Locke and Tartoff, 1994); arm^{S6} and arm^{XM19} (Peifer and Wieschaus, 1990; Orsulic and Peifer, 1996a); and arm^{TD} (Peifer and Wieschaus, 1990). wg^{IG22} (Lindsley and Zimm, 1992). arm^{S10} (Pai et al., submitted) is under the control of the GAL4-UAS (Brand and Perrimon, 1993) and driven by the ubiquitously expressed GAL4 line e22c. The GAL4-regulated *dTCF* and *dTCFΔN* transgenes were created by cloning *dTCF* or an N-terminal 31–amino acid deletion thereof into the pUAST vector (Brand and Perrimon, 1993; details available on request). *dTCF* rescue constructs, called *P[w+, hsdTCF+]*, were generated by inserting a cDNA clone encoding amino acids 1–695 of dTCF into pCaSpeR-hs (Thummel et al., 1988). Both GAL4-driven and rescue constructs were coinjected with p25.7wc DNA into embryos prepared by standard protocols (Spradling, 1986). For the GAL4-driven constructs, multiple lines were obtained. For the rescue construct, a single original line was obtained (*P[w+,hsdTCF+]15–2*). Additional inserts, *P[w+,hsdTCF+]15–2*, *P[w+,hsdTCF+]15–32*, and *P[w+,hsdTCF+]15-X*, were obtained by mobilization with the transposase source 2–3 (99B) (Robertson et al., 1988). Engrailed was detected using monoclonal anti-engrailed, as described in DiNardo et al. (1985).

In Situ Hybridization

Digoxigenin whole-mount in situ hybridization (Tautz and Pfeifle, 1989) was performed. A 700 bp XhoI–HindIII cDNA fragment was prepared and labeled as described (Ray et al., 1991). A ribosomal protein clone and pKS were also labeled and hybridized in parallel as positive and negative controls, respectively.

Generation of Constructs

For transfection studies, *dTCF* cDNA was subcloned into the eukaryotic expression vector pCDNA. The Armadillo mutants arm^{S8} and arm^{XM19} were generated by PCR. PCR fragments were cut with SacI and EcoRI and used to replace a SacI–EcoRI fragment in pCS2-ARM, generating a stop codon at amino acids 750 (arm^{S8}) and 681 (arm^{XM19}), respectively. For the GAL4 fusion protein studies, the relevant PCR fragments were cloned into BamHI–EcoRI– digested pJ3ω (van de Wetering et al., 1993). The fusion proteins start at comparable positions in the last arm repeat, specifically, S^{694} in Armadillo and S^{682} in β-catenin. Oligonucleotides were from Isogen (Maarssen, the Netherlands).

Site Selection

A PCR fragment encoding the dTCF-A HMG box (amino acids 247–362) was subloned into pET 21c (Novagen) and transformed into Escherichia coli BL21(DE3). The His-tagged protein was purified using Ni^{2+}-coated resin (New England Biolabs). For the site selection procedure, a random probe was generated by annealing ^{32}P-labeled primer A (GTTACCGCGGATCCGAATTCCC) to (CTCGGTACCTCGA GTGAAGCTTGA$(N)_{18}$GGGATTCGGATCCGC GGTAAC, followed by extension with Klenow fragment and deoxynucleoside triphosphates. After gel retardation (van de Wetering et al., 1991), the retarded probe was PCR-reamplified using primers A and B (CTCGGTACCTCGAGTGAAGCTTGA) in the presence of [^{32}P]dCTP and used to repeat the experiment. After nine rounds of selection, probe fragments were subcloned. The sequences of 36 independent fragments, as identified by differences in the flanking sequences, were compiled.

Yeast Two-Hybrid Assays

These assays were performed essentially as described (Pai et al., 1996). pCK2 (encoding fusions to the LexA DNA-binding domain), pCK4 (encoding fusions to the activation domain of GAL4), and their Armadillo derivatives (Pai et al., 1996) were used. The indicated dTCF and XTcf-3 fragments were cloned into pCK2 or pCK4. Yeast strain L40 was transformed and double-selected on Trp^{-}, Leu^{-} plates. Activation of the His reporter was assayed on Trp^{-}, Leu^{-}, His^{-} plates supplemented with 3-aminotriazole. Liquid β-galactosidase assays were carried as described (Pai et al., 1996). Assays were performed on at least six independent transformants in duplicate for each Armadillo/TCF pair.

Cat Assays and Luciferase Assays

Described in detail elsewhere (van de Wetering et al., 1991). In short, 2×10^6 IIAI.6 B cells were transfected by electroporation with a total of 7 μg of the various combinations of plasmids: 1 μg of CAT reporter plasmid; 2 μg of TCF factor expression vectors; 4 μg of β-catenin/Armadillo expression vector, and empty pCDNA vector as stuffer. CAT vectors were (pTK(56)7 and pTK(56Sac)7 (van de Wetering et al., 1991). cDNAs encoding tagged versions of β-catenin, Armadillo, dTCF, hTCF-1, hLEF-1, and XTcf-3 were inserted into the mammalian expression vector pCDNA. After 48 hr, CAT values were determined as pristane/xylene–extractable radiolabeled, butyrylated chloramphenicol. An oligonucleotide containing three copies of the binding site (CCTTTGATC) or a mutant thereof (CCTTTGGCC), cloned into a blunted XbaI site of pcFos-luciferase, yielded pTOPFLASH (optimal motif) and pFOPFLASH (mutant motif) (details available on request.) Transfections were the same as for the CAT assay. Cells were harvested after 16 hr and lysed in 1 mM DTT, 1% Triton X-100, 15% glycerol, 25 mM Tris (pH 7.8), and 8 mM $MgCl^2$. Luciferase activity was determined on a Lumac/3M biocounter.

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